Metabolism of Nicotine by Human Liver Microsomes: Stereoselective Formation of *trans*-Nicotine *N'*-Oxide

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Liver microsomes from humans catalyze the NADPH-dependent oxidation of (*S*)-nicotine. The principal product is the 5'-carbon atom oxidation product, nicotine $\Delta^{1',5'}$-iminium ion, which is efficiently converted to the γ-lactam derivative cotinine in the presence of aldehyde oxidase. Another major product is nicotine *N'*-oxide. In contrast to previous reports describing in vitro or in vivo studies, formation of only *trans*-nicotine *N'*-oxide was observed. Demethylation of nicotine was not observed. Studies on the biochemical mechanism of nicotine 5-carbon atom oxidation strongly implicate one major cytochrome P-450 isoenzyme (i.e., P-450 2A6) as largely responsible for $\Delta^{1',5'}$-iminium ion formation. Stereoselective formation of *trans*-nicotine *N'*-oxide may be catalyzed in large part by the flavin-containing monooxygenase (form II). These conclusions are based on the effects of alternate substrates for the flavin-containing monooxygenase, heat inactivation studies, immunoblot studies, and selective substrates for cytochromes P-450. The results suggest that (*S*)-nicotine trans N'-oxygenation and $\Delta^{1',5'}$-iminium ion formation may be selective probes of human liver flavin-containing monooxygenase form II and cytochrome P-450 2A6 activities, respectively, useful for in vivo phenotyping of humans.

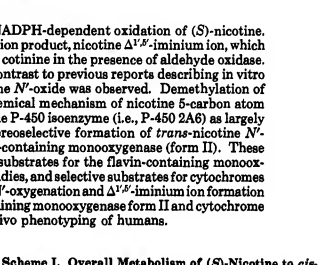
Introduction

Nicotine is one of the most widely used psychoactive drugs in the world, and almost 30% of adult Americans smoke tobacco despite convincing knowledge of the health hazards of smoking (*1*). Many of the pharmacological effects of smoking tobacco are due to nicotine. Nicotine causes complex central nervous system, behavioral, cardiovascular, endocrine, neuromuscular, and metabolic effects in humans (*2*). Although nicotine itself is not carcinogenic, nitroso derivatives of nicotine and other nicotine metabolites are carcinogenic (*3*, *4*). Whether nicotine-derived nitrosamines are the causative agents of cancer in humans exposed to tobacco is unknown, but what is clear is that nicotine is (bio)transformed to highly reactive materials which covalently modify proteins (*5–7*) and DNA (*8–10*).

At present, about 80–90% of a dose of nicotine that is consumed can be accounted for in terms of identified human urinary metabolites (*11*). The major excreted human urinary metabolites of nicotine are cotinine (10–15%), nicotine *N'*-oxide (4%), and *trans*-3'-hydroxycotinine (20%–30%) (Scheme I) (*12*). In 26 smokers, mean cotinine excretion was 1.39 mg/24 h, while nicotine *N'*-oxide was excreted at a rate of 0.56 mg/24 h in the same smokers (*13*).

The two major monooxygenases that metabolize nicotine are the cytochromes P-450 (P-450) and the flavin-containing monooxygenases (FMO).[1] In animals, P-450 2B1[2] (i.e., the major form induced by phenobarbital) has been most often implicated as the putative nicotine oxidase (*14*, *15*) primarily responsible for nicotine $\Delta^{1',5'}$-iminium ion formation (*7*, *14*). Thus, liver microsomes from

Scheme I. Overall Metabolism of (*S*)-Nicotine to *cis*- and *trans*-Nicotine *N'*-Oxide, Cotinine, and Nornicotine



[1] Abbreviations: FMO, flavin-containing monooxygenase; DETAPAC, diethylenetriaminepentaacetic acid; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CI, chemical ionization; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; PTLC, preparative thin-layer chromatography; CD, circular dichroism; NOE, nuclear overhauser effect; GC/MS, gas chromatography/mass spectrometry.

[2] Cytochrome P-450 nomenclature from Nebert et al. [(1991) *DNA Cell Biol.* 10, 1–14]. An alternate nomenclature for FMO form I (FMO 1A1) and FMO form II (FMO 1D1) has been proposed based on the rabbit liver enzymes.

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rats and rabbits pretreated with phenobarbital as compared to microsomes from untreated animals showed an increased covalent binding of reactive nicotine metabolites and cotinine formation (in microsomes supplemented with aldehyde oxidase). However, in human liver, the P-450 enzyme analogous to the phenobarbital-inducible form present in rat liver does not appear to be present to a significant extent, although barbiturates have been shown to induce P-450 3A in man.

In contrast to P-450, little is known concerning FMO-dependent human drug metabolism (*16*, *17*). The N-oxygenation of trimethylamine and nicotine has been proposed to be primarily dependent on FMO (*18*, *19*) although the data are less than conclusive. Hog liver FMO efficiently N'-oxygenates nicotine, but the diastereoselectivity reported for this reaction was quite low (*20*). Adult human liver microsomes are capable of FMO-related N- and S-oxygenation activity although notable substrate specificity differences between hog liver and human liver FMO were proposed (*21*–*23*). The diastereoselectivity for human liver microsomal FMO-dependent nicotine N'-oxygenation is unknown. In addition, the diastereoselectivity of nicotine N'-oxide N-oxygenation has not been described.

In this report, we describe studies on the biotransformation of nicotine and nicotine N'-oxide diastereomers by human liver microsomes. Nicotine is efficiently oxidized to the N'-oxide and to the $\Delta^{1'5'}$-iminium ion (and oxidized to cotinine by aldehyde oxidase) by human liver microsomal preparations. Studies on the molecular basis for formation of nicotine metabolites suggest that human liver FMO form II and P-450 2A6 primarily catalyze the N'-oxide and $\Delta^{1'5'}$-iminium ion formation, respectively. That nicotine N,N'-oxide is stable to the metabolic incubation conditions and is not further metabolized suggests that nicotine N'-oxide stereochemistry is a direct indicator of monooxygenase action.

Experimental Procedures

Chemicals. Chemicals used in this study were of the highest purity available. (*S*)-Nicotine, (*S*)-cotinine, and (*S*)-nornicotine were synthesized as previously described (*24*). (*S*)-Nicotine N'-oxide was prepared by the method of Taylor and Boyer (*25*). Chlorpromazine, chlorpromazine sulfoxide, chlorpromazine side chain N'-oxide, and desmethyl chlorpromazine were obtained from Dr. A. A. Manian (National Institutes of Mental Health, Rockville, MD). The purity of these compounds was identical to that previously described (*26*). Thioures, thiobenzamide, metachloroperbenzoic acid, and other chemicals were purchased from Aldrich Chemical Co., (Milwaukee, WI). Other reagents, buffers, and solvents were from Fisher Chemical Co. (San Jose, CA). All of the compounds of the NADPH-generating system were obtained from Sigma Chemical Co., (St. Louis, MO). Chromatography was done on Silica Woelm (70–150 mesh, Universal Scientific, Inc.). Preparative TLC was done with 20- × 20-cm cellulose microcrystalline PK2F (1000-μm thickness) from Whatman (Clifton, NJ).

Instrument Analysis. ^{1}H NMR spectra were recorded with a General Electric spectrometer operating at 300 MHz. ^{1}H chemical shifts were expressed in ppm downfield from tetramethylsilane. Chemical ionization (CI) mass spectra were recorded with a VG 70S spectrometer set at 6 kV. Circular dichroism spectra were recorded on a Jasco J-500A spectropolarimeter.

Synthesis of (*S*)-Nicotine N'-Oxide. Nicotine N'-oxide was synthesized by a method previously described (*25*). This procedure produced an approximate 4:1 mixture of 1'(*S*),2'(*S*)-trans:1'(*R*),2'(*S*)-cis diastereomers of nicotine N'-oxide as determined by ^{1}H NMR and HPLC (Figure 1). The diastereomers of nicotine N'-oxide (10 mg) were separated on cellulose PTLC (eluent 1 butanol/2-propanol/ammonium hydroxide, 50:25:25 v:v) to give 6.8 mg of *trans*-nicotine N'-oxide with an R_f value of 0.58 and 1.6 mg of cis N'-oxide with an R_f value of 0.53: ^{1}H NMR ($CDCl_3$) (*trans*-nicotine N'-oxide) δ 2.0–2.10 (m, 1 H, C^4H), 2.25–2.30 (m, 1 H, C^4H), 2.45–2.56 (m, 1 H, C^3H), 2.56–2.75 (m, 1 H, C^3H), 2.93 (s, 3 H_a N'-CH_3), 3.56 (ddd, 1 H, J = 20.1, 9.3, and 1.2 Hz, C^2H), 3.74 (ddd, 1 H, J = 10.5, 9.3, and 2.1 Hz, C^2H), 4.20 (dd, 1 H, J = 7.5 and 11.4 Hz, C^2H), 7.32 (dd, 1 H, J = 7.8 and 4.8 Hz, C^5H), 8.20 (dt, 1 H, J = 7.8 and 1.8 Hz, C^4H), 8.53 (d, 1 H, J = 1.8 Hz, C^2H), 8.59 (dd, 1 H, J = 4.8 and 1.8 Hz, C^6H); (*cis*-nicotine N'-oxide) δ 1.95–2.10 (m, 1 H, C^4H), 2.17–2.40 (m, 1 H, C^4H), 2.43–2.60 (m, 1 H, C^3H), 2.70–2.85 (m, 1 H, C^3H), 2.77 (s, 3 H_a N'-CH_3), 3.60–3.78 (m, 2 H_a, C^2H_2), 4.70 (t, 1 H, J = 8.4 Hz, C^2H), 7.36 (dd, J = 8.1 and 4.8 Hz, C^5H), 7.93 (dt, 1 H, J = 8.1 and 1.8 Hz, C^4H), 8.65 (dd, 1 H, J = 4.8 and 1.8 Hz, C^6H), 8.71 (d, 1 H, J = 1.8 Hz, C^2H). Mass spectral data (CI): calcd, MH^+ 179.1103; found, *m/z* (relative intensity) (*trans*-nicotine N'-oxide) 179.1170 (MH^+, 91.3, $C_{10}H_{15}N_2O$), 163.1213 ($MH^+ - O$, 100, $C_{10}H_{15}N_2$), 162.1110 ($M^+ - O$, 23.9, $C_{10}H_{14}N_2$), 161.1067 ($MH^+ - H_2O$, 85.6, $C_{10}H_{13}N_2$), 133.0757 (10.4), 119.0688 (23), 84.1449 (49.9); (*cis*-nicotine N'-oxide) 179.1156 (MH^+, 17.4, $C_{10}H_{15}N_2O$), 163.1265 ($MH^+ - O$, 100, $C_{10}H_{15}N_2$), 162.1157 ($M^+ - O$, 15, $C_{10}H_{14}N_2$), 161.1117 ($MH^+ - H_2O$, 35.4, $C_{10}H_{13}N_2$), 133.0761 (12.5), 119.0679 (14), 84.1470 (43).

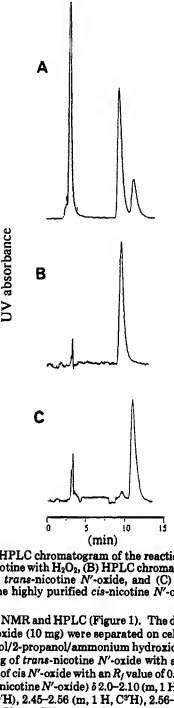

Figure 1. (A) HPLC chromatogram of the reaction product of treating (*S*)-nicotine with H_2O_2, (B) HPLC chromatogram of the highly purified *trans*-nicotine N'-oxide, and (C) HPLC chromatogram of the highly purified *cis*-nicotine N'-oxide.

Synthesis of (*S*)-Nicotine *N,N'*-Dioxide. To a stirred solution of metachloroperbenzoic acid (1.9 g, 11 mmol) in 25 mL of CH_2Cl_2 was added slowly (*S*)-nicotine (0.8 mL, 5 mmol) in 25 mL of CH_2Cl_2 at 0 °C. The solution was stirred at 0 °C for 2 h. The reaction was extracted twice with 25 mL of H_2O. The aqueous solution was washed twice with 25 mL of diethyl ether and dried to give a yellow oil. The product was purified on a preparative TLC plate (silica gel GF, 1000 μm, Analtech, Newark, DE) eluted twice with methanol containing 1% ammonium hydroxide to give 737 mg of (*S*)-nicotine *N,N'*-dioxide (76% yield); ^1H NMR and HPLC analysis indicated that the ratio of trans to cis *N,N'*-oxide was 6:1.

(*S*)-Nicotine *N,N'*-dioxide: ^1H NMR ($CDCl_3$) δ 1.87 (m, 1 H, C^3H), 2.09 (m, 1 H, C^3H), 2.29 (m, 2 H, C^4H), 2.83 (s, 3/4 H, cis N-CH_3), 3.12 (s, 15/4 H, trans N-CH_3), 3.54 (m, 2 H, C^5H), 4.16 (m, 1 H, C^2H), 7.11 (m, 1 H, Pyr H), 7.52 (m, 1 H, Pyr H), 7.95–8.05 (m, 1 H, Pyr H), 8.31 (s, 1/4 H, trans Pyr H), 8.39 (s, 1/4 H, cis Pyr H). Mass spectral data (HREI+): calcd, M^+ 194.1055 ($C_{10}H_{14}N_2O_2$); found, *m/z* (relative intensity) 194.1062 (M^+,24.9), 178.1108 ($M^+ - O$, 16.4) 176.0948 ($M^+ - H_2O$, 29.1), 162.1149 ($M^+ - O_2$, 12.8) 160.0985 ($M^+ - H_2O_2$, 39.5), 159.094 ($M^+ - H_3O_2$, 59.7) 135.0664 (34.1), 118.0651 (100).

Liver Specimens and Microsome Preparation. The human liver specimens were obtained under a protocol approved by the Committee for the Conduct of Human Research at the Medical College of Wisconsin. Liver microsomal fractions were isolated according to the method described before (*27*), and the specific content of microsomal P-450 protein concentration and the P-450 content were determined as previously described (*28, 29*). Each microsome sample has been thoroughly characterized for all of the major human liver cytochrome P-450s (see ref 34 for details) and FMO isozymes (see below). The supernatant fraction of rat liver microsomes from untreated Sprague-Dawley rats was obtained from the 100000*g* spin during a microsome preparation (*7*).

Incubation Systems. A typical incubation mixture (final volume 0.25 mL) contained 50 mM potassium phosphate buffer (i.e., pH 7.4 for cotinine determinations and pH 8.4 for nicotine *N'*-oxide determinations), 0.5 mM $NADP^+$, 2.0 mM glucose 6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase, 4.3 mg of protein of the rat liver supernatant fraction (as a source of aldehyde oxidase), 0.4 mg of microsomal protein, and 0.6 mM DETAPAC. The reaction was initiated by the addition of substrate and incubated at 37 °C with shaking in air. At various time intervals, the reaction was stopped by the addition of 3 volumes of cold 2-propanol/CH_2Cl_2 (2:1 v:v) after saturation with Na_2CO_3, centrifuged, and then analyzed for products by the HPLC procedure given below.

The profile of nicotine metabolites was determined by HPLC analysis of 2-propanol/CH_2Cl_2 extracts of the reaction mixtue. For nicotine *N'*-oxides, the metabolic products from the extracts were separated and quantified by an IBM Model 9533 HPLC interfaced to a HP Model 3392A integrator with a UV detector set at 260 nm, fitted with a Partisil 10-PAC analytical column (25 cm × 0.4 cm) from Whatman (Clifton, NJ) (*30*). This column was a generous gift of Professor John Thompson (University of Colorado). For the separation of nicotine *N'*-oxide diastereomers, the mobile phase consisted of an isocratic system set at 5% water and 95% methanol. This system efficiently separated nicotine, *trans*-nicotine *N'*-oxide, and *cis*-nicotine *N'*-oxide, which had retention volumes of 4.2, 9.6, and 11.0 mL, respectively. Metabolites were quantified by comparing the metabolite and substrate peak areas of the chromatogram. The recovery of material as judged by HPLC was >90% (using an internal standard of 5-methylnicotine), and >95% of this material was nicotine or *trans*-nicotine *N'*-oxide.

For analyses of cotinine, the 2-propanol/CH_2Cl_2 extracts (saturated with Na_2CO_3) were separated and quantified by an IBM Model 9533 HPLC as above fitted with a 5-μm, 25-cm × 4.5-mm AXXIOM silica analytical column (Richard Scientific, Novato, CA) (*31*). The mobile phase consisted of 2-propanol/CH_3OH/CH_3CN/HClO$_4$ (18:59:23:0.018 v:v). This system efficiently separated cotinine, nornicotine, and nicotine, which have retention volumes of 6.1, 7.2, and 7.9 mL, respectively. Metabolites were quantified by comparing the metabolite and substrate peak areas of the chromatogram.

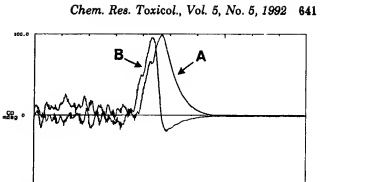

Figure 2. Circular dichroism spectra for highly purified *trans*-nicotine *N'*-oxide (**A**) and *cis*-nicotine *N'*-oxide (**B**).

For analyses of nicotine *N,N'*-dioxide the metabolic incubations were stopped by addition of cold methanol (0.25 mL) and the mixture was centrifuged. The supernatant was directly analyzed by HPLC as described above with a Partisil 10-PAC analytical column. The mobile phase consisted of methanol/water (95:5 v:v). This system efficiently separated nicotine, *trans*-nicotine *N'*-oxide, *cis*-nicotine *N'*-oxide, *trans*-nicotine *N,N'*-dioxide, and *cis*-nicotine *N,N'*-dioxide, which had retention volumes of 9.6, 11.0, 18.9, and 22.9 mL, respectively.

Assays. Microsome monooxygenase activities and immuno cross-reactivity were determined as described below. P-450 coumarin 7-hydroxylase activity was determined exactly as described previously (*32*). Human liver microsomes had significant levels of chlorpromazine N- and S-oxygenation activities. FMO-dependent chlorpromazine N-oxygenation and P-450-dependent chlorpromazine S-oxygenation was assayed in human liver microsomes by a modification of a procedure previously described (*33*) and quantified by HPLC as described before (*31*).

Antisera and Immunoblotting. Antibodies that recognized specific human P-450s were characterized as described previously (*34*). Antibodies to hog and guinea pig liver FMO were raised in rabbits. The antibody to guinea pig liver FMO was a generous gift of Drs. K. Oguri and H. Yamada (Kyushi University, Fukuoka, Japan). The hog liver IgG fraction was fractionated from antisera by DEAE-Sepharose chromatography (*35*).

Results

Chemistry. The chemical synthesis of *cis*- and *trans*-nicotine *N'*-oxide diastereomers was done to provide sufficient material for the determination of the absolute configuration of these materials and to provide materials to identify nicotine metabolites in vitro. Treatment of nicotine with hydrogen peroxide resulted in a 4:1 mixture of *trans*- and *cis*-nicotine *N'*-oxide. Surprisingly, treatment of nicotine with the modified Sharpless oxidation reagent under conditions which generally produced high diastereoselectivity (*36*) (with similar compounds) did not result in detectable levels of nicotine *N'*-oxide formation (data not shown). *cis*- and *trans*-nicotine *N'*-oxides were separated by PTLC. As shown in Figure 1A, the HPLC chromatogram of the crude synthetic nicotine *N'*-oxide product (in a ratio of 4:1, trans:cis) was separated into nearly pure (≥98%) individual trans (Figure 1B) and cis (Figure 1C) diastereomers after PTLC. Each purified diastereomer was completely characterized by spectral means. The individual CD spectra of purified *cis*- and *trans*-nicotine *N'*-oxide are shown in Figure 2: *trans*-nicotine *N'*-oxide has a positive Cotton effect (Figure 2A), and *cis*-nicotine *N'*-oxide possessed both a positive and a

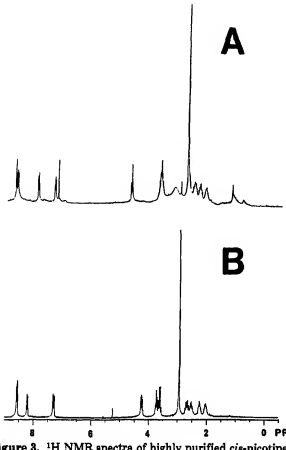

Figure 3. ^{1}H NMR spectra of highly purified *cis*-nicotine N'-oxide (A) and *trans*-nicotine N'-oxide (B).

Table I. Differential Nuclear Overhauser Effect Studies for *trans*- and *cis*-Nicotine N'-Oxide

compound[a]	position irradiated[b]	signal observed (% NOE)
trans-nicotine N'-oxide	C^2H	N'-CH_3 (3.4)
		C^2H (5.5)
		C^4H (3.0)
		C^5H (0.8)
	N'-CH_3	C^2H (0.5)
cis-nicotine N'-oxide	N'-CH_3	C^2H (11.3)
		C^6H (13.2)
		C^4H (9.4)
		C^5H (5.7)

[a] Each compound was ≥98% pure as judged by HPLC (shown in Figure 1). [b] The ^{1}H NMR NOE studies were done as described in the Experimental Procedures.

negative Cotton effect (Figure 2B). The identity of the purified nicotine N'-oxide diastereomers was further examined by ^{1}H NMR. The prominent resonaces were consistent with the structure of *cis*-nicotine N'-oxide (Figure 3A). Figure 3B shows the ^{1}H NMR spectra of *trans*-nicotine N'-oxide. To confirm the chemical structures of each purified diastereomer, NOE studies were carried out. The values of the difference ^{1}H NOE on *trans*-nicotine N'-oxide are shown in Table I. An NOE between the C^2 proton of the pyrrolidine ring and the N-methyl group was observed when the latter was irradiated. No NOE effect was observed on the pyridyl protons when the N-methyl group was irradiated. In contrast, *cis*-nicotine N'-oxide showed strong NOE effects on all of the pyridyl protons when the N-methyl group was irradiated. The results strongly suggest that the nicotine N'-oxide diastereomer with retention volumes of 9.6 and 11.0 mL corresponds to *trans*- and *cis*-nicotine N'-oxide, respectively.

Identification of Nicotine Metabolites. The metabolism of nicotine in the presence of human liver microsomes was done in order to determine the nature of the products and the identity of the monooxygenase(s) responsible for the biotransformation. Aerobic incubation of human liver microsomes with nicotine in the presence of NADPH resulted in formation of nicotine N'-oxide and cotinine. Under the experimental conditions of the reaction, formation of nornicotine was not observed. Formation of nicotine N'-oxide and cotinine was markedly pH-dependent. Thus, nicotine N'-oxide formation was much more efficient at pH 8.4 than pH 7.4 although significant amounts of cotinine were formed at both pH 7.4 and pH 8.4. The results suggested that each product was formed in a distinct reaction catalyzed by a different monooxygenase. To confirm the chemical nature of the metabolic products, organic extracts of metabolic incubations with human liver microsomes were analyzed by GC/MS. The mass spectral data [i.e., m/z (relative intensity) = 178 (14.2), 119 (100), 118 (76.1), and 60 (96.8) and m/z (relative intensity) = 176 (35.9), 147 (12.5), 118 (19.6), and 98 (100)] as well as the fragmentation pattern for nicotine N'-oxide (analyzed as the oxazine, see ref 13) and cotinine, respectively, of these organic extracts were identical with that of authentic *trans*-nicotine N'-oxide and cotinine. The determination of the structure of the metabolites, however, did not provide direct evidence for the identity of the monooxygenase responsible for each product.

To identify the monooxygenases responsible for nicotine metabolism in human liver microsomes, we utilized the reported differences in pH optima of the two monooxygenases, cytochromes P-450 (P-450) and the FMO. Thus, human liver P-450 and FMO have reported pH optima of pH 7.4 (*37*, *38*) and pH 9.0 (*21*), respectively. In the presence of NADPH, preliminary experiments showed that nicotine N'-oxide formation was optimal at pH 8.4. At pH 7.4, the rate of nicotine N'-oxide was decreased. The results were consistent with a primary role of FMO for nicotine N'-oxide formation and for a role of P-450 in the formation of nicotine $\Delta^{1',5'}$-iminium ion (which is rapidly converted to cotinine in the presence of aldehyde oxidase). The metabolism of nicotine to nicotine N'-oxide and nicotine $\Delta^{1',5'}$-iminium ion had the same pH optima as marker activities for human liver FMO II (i.e., chlorpromazine N-oxygenation) and cytochromes P-450 (i.e., chlorpromazine S-oxidation), respectively.[3] However, the results do not rule out the involvement of cytochromes P-450 in nicotine N'-oxide formation.

To provide further support for a role of FMO and P-450 in nicotine N'-oxide and cotinine formation, respectively, the molecular basis for product formation was examined with a variety of metabolic incubation conditions. Nicotine N'-oxide formation was almost completely dependent upon NADPH and active microsomal protein (Table II). At pH 7.0, thiourea (*39*) and thiobenzamide (*40*), two well-documented alternative substrate competitive inhibitors of hepatic FMO, completely abolished nicotine N'-oxygenase activity. At pH 8.4, nicotine N'-oxygenase activity was increased significantly and thiobenzamide completely inhibited and thiourea partially inhibited nicotine N'-ox-

[3] J. R. Cashman and S. E. Wrighton, unpublished results.

Table II. Effect of Inhibitors on Nicotine Oxidation by Human Liver Microsomes

incubation conditions	nicotine N'-oxide formation[a] [(pmol/(min·mg of protein)] pH 7.0	pH 8.4	cotinine formation pH 7.0
complete system[b]	24.6 ± 8.3	190.6 ± 46.5	1425 ± 136.2
omit NADPH	ND[c]		10.0 ± 9.9
omit protein	ND	9.7 ± 4.5	ND
heat-inactivated protein[d]	ND	ND	954.8 ± 44.5
+thiourea (1 mM)	ND	19.4 ± 11.2	1460.3 ± 187.2
+thiobenzamide (1 mM)	ND	ND	1757.7 ± 313.2

[a] The only product detected was the trans N'-oxide. [b] The complete system contained 50 mM potassium phosphate, pH 7.0 or 8.4, the NADPH-generating system, 0.5 mM nicotine, 0.6 mM DETAPAC, 4.3 mg of rat liver microsome supernatant, and 0.4 mg of microsomal protein in a final volume of 0.25 mL. The product was determined by the HPLC procedure described in the Experimental Procedures. The results are mean values of three determinations using pooled microsomes ±SD. [c] ND, not detected; the limit of detection was approximately 5 pmol/(min·mg of protein). [d] Microsomal protein was heated at 55 °C for 1 min in the absence of NADPH and cooled, 2 μg of catalase and the NADPH system was added, and the reaction was carried out as normal.

ygenase activity. Under conditions of heat inactivation which generally abolished FMO activity (*22*) and left P-450 activity intact, nicotine N'-oxygenase activity was completely eliminated although almost 70% of cytochrome P-450-dependent cotinine formation activity was retained. Nicotine $\Delta^{1',5'}$-iminium ion formation (as measured by conversion to cotinine) was significant at pH 7.0 and was strongly dependent upon NADPH and active microsomal protein. Thiourea and thiobenzamide did not inhibit cotinine formation (Table II).

Role of FMO in Nicotine N'-Oxide Formation. To examine a role of human liver FMO in nicotine N'-oxygenation, the relative rate of N'-oxide formation was determined in human liver microsome preparations A–N at pH 8.4. In addition, the stereoselectivity of nicotine N'-oxide formation was also examined. Formation of nicotine N'-oxide was not dependent on the gender, age, smoking history, or previous drug administration history of the subject that the microsome preparation was obtained from (Table III). As suggested by previous animal (*41*) and human (*21*, *22*) liver studies, microsomes obtained from subjects treated with barbiturates or ethanol (i.e., C, E, F, I, K, M, N) did not show any clear pattern of FMO enzyme induction, and in fact, microsomes from subjects without a history of previous drug administration (i.e., samples A and J) formed among the highest amount of nicotine N'-oxide observed. However, as noted previously (*21*) considerable interindividual variation of monooxygenase activity among hepatic samples has been observed.

To assess the relative amount of FMO present in microsomal preparations A–N, immunoblots were performed with antibodies directed against hog liver FMO (form I) and guinea pig liver FMO (form II) (*42*). Antibodies directed against hog liver FMO detected a protein with ca. M_r = 60 000 in all the human liver microsomal samples, but the signal was very weak (data not shown). In contrast, a strong signal was observed with antibodies directed against guinea pig liver FMO (form II), and in some cases the strongest signal (i.e., samples G and J–L) corresponded to high levels of nicotine N'-oxygenase activity. However, there were a few cases of microsome samples with moderate levels of immunoreactive protein that corresponded to relatively high rates of nicotine N'-oxygenation activity (i.e., A, C, E, and N) (Table III). The results were consistent with a prominent role for FMO in nicotine N'-oxygenation but did not rule out the involvement of other monooxygenases in the N'-oxygenation of nicotine.

To examine the possible involvement of additional monooxygenases in nicotine N'-oxygenation, the stereoselectivity for each metabolic incubation (i.e., samples A–N) was determined. Surprisingly, in all 14 cases examined, the stereoselectivity of the reaction gave exclusive formation of *trans*-nicotine N'-oxide. This result was quite different than the one determined previously for the N'-oxidation of nicotine by highly purified hog liver FMO (form I) or guinea pig microsome preparations (i.e., reported formation of 51:49 and 79:21 *cis*-:*trans*-nicotine N'-oxide, respectively) (*20*). It is unlikely that any cis: trans isomerization occurred during the short period of the metabolic incubation or during the extraction and analyses because addition of pure nicotine N'-oxide diastereomers or mixtures of N'-oxide diastereomers did not result in alteration of the stereochemistry previously determined. In addition, the sensitivity and reproducibility of the assay precluded even minor amounts of *cis*-nicotine N'-oxide from being undetected (see Experimental Procedures).

Role of P-450 in the Formation of Cotinine. The role of human liver P-450 in the microsomal metabolism of nicotine to nicotine $\Delta^{1',5'}$-iminium ion was examined in vitro at pH 7.4 by coupling iminium ion formation to cotinine production by the action of aldehyde oxidase (i.e., the 100000*g* supernatant fraction of rat liver microsomes). Under the conditions of the metabolic incubations, no detectable amount of nornicotine as detected, and we estimate that the ratio of cotinine:nornicotine formed for all of the human liver microsome samples examined was probably greater than 97:3. Presumably, prolonged metabolic incubation in the presence of considerably larger amounts of protein could have produced detectable amounts of nornicotine, but limitations on the availability of human liver microsomes precluded any such studies. In agreement with these data, however, is the report that nornicotine is a minor in vivo metabolite in humans administered nicotine (*43*).

In contrast to nicotine N'-oxide formation, production of nicotine $\Delta^{1',5'}$-iminium ion showed some dependence upon the previous drug administration history of the subject from which the hepatic microsomes were isolated. That is, two of the highest rates of iminium ion formation occurred in the presence of human liver microsomes from barbiturate-pretreated subjects (i.e., E and I) (Table III). Because human hepatic P-450 3A forms are induced by barbiturates, the microsomes (i.e., A–N) were characterized by immunological means to correlate the rate of formation of nicotine $\Delta^{1',5'}$-iminium ion with P-450 composition and amount. Of the seven major P-450 enzymes examined (i.e., 1A2, 2A1, 2C8, 2C9, 2D6, 2E1, 3A5, and 3A total) the greatest linkage between immunoreactivity and nicotine $\Delta^{1',5'}$-iminium ion formation was observed for P-450 2A6 (r = 0.84) (Table III). The results are in agreement with previous studies which showed that phenobarbital increased the amount of P-450 2A6 2–3-fold in human hepatocytes in primary culture (*37*). Like nicotine N'-oxygenation, neither gender, age, nor smoking history provided a direct relation between nicotine $\Delta^{1',5'}$-iminium ion formation and P-450 2A6 levels. Furthermore, the P-450 2A6-catalyzed 7-hydroxylation of coumarin correlated well with the ability of the microsomes to produce

Table III. Oxidation of Nicotine and Coumarin by Human Liver Microsomes[a]

patient code	gender	age	drug history	FMO (form II) immunoreactivity[b]	metabolite formed [pmol/(min·mg of protein)] nicotine N′-oxygenation[c]	cotinine	nornicotine[b]	P-450 3A immunoreactivity[d]	metabolite formation 7-OH coumarin [pmol/(min·mg of protein)]	P-450 2A6 immunoreactivity[d]
A	M	25	none[e]	3	226	294	ND	100	330	100
B	M	50	none[e]	2	88	238	ND	110	280	143
C	M	22	ethanol[f]	2	181	187	ND	118	230	139
D	M	31	none[e]	1	54	568	ND	73	740	389
E	M	14	pentobarb[g]	2	180	376	ND	262	600	355
F	F	50	ethanol[f]	2	55	353	ND	182	520	395
G	F	48	teldrin[f]	5	225	299	ND	142	410	264
H	F	28	none[f]	2	34	284	ND	73	330	192
I	M	43	phenobarb[g]	3	45	750	ND	315	780	718
J	F	55	none[f]	4	133	377	ND	121	360	139
K	M	23	ethanol[f]	4	192	431	ND	92	940	569
L	F	58	dopamine[f]	4	141	208	ND	66	110	31
M	M	18	ethanol[f]	3	67	234	ND	86	400	224
N	M	21	ethanol[f]	2	127	311	ND	98	430	132

[a] Incubations were performed with 0.4–2.0 mg of microsomal protein, 4.3 mg of rat liver microsome supernatant (as a source of aldehyde oxidase), 0.5 mM substrate, and 0.5 mM NADPH at pH 8.4 and 37 °C for 10–20 min, and products were quantified by HPLC. For cotinine, nornicotine, and 7-OH-coumarin formation, incubations were at pH 7.4. Values are the mean of 2–4 determinations ± 10%. [b] Relative immunoquantitation; 5 = greatest, 1 = weakest. [c] Substrate oxygenation was a linear function of protein (0–2.0 mg) and time (0–20 min) and was strictly dependent on NADPH. [d] 75 smoking pack years. [e] 35 smoking pack years. [f] Unknown smoking history. [g] Nonsmoker. [h] In all samples examined only formation of cotinine and nicotine N′-oxide was observed: ND, Not Detectable, limit of detection 10 pmol/(min·mg of protein). [i] Relative percent immunoquantitation was determined with human P-450 form-selective antibodies (34).

the nicotine $\Delta^{1',5'}$-iminium ion (r = 0.80). Because P-450 3A levels were elevated in the two samples from patients receiving barbiturates, a reasonable correlation was observed between $\Delta^{1',5'}$-iminium ion formation and P-450 3A levels (r = 0.59). However, when the two liver samples from the barbiturate-treated patients were removed from the correlation, no relationship was observed between P-450 3A levels and nicotine $\Delta^{1',5'}$-iminium ion formation (r = −0.03) whereas an excellent correlation was still observed between nicotine $\Delta^{1',5'}$-iminium ion formation and P-450 2A6 levels (r = 0.69) and coumarin 7-hydroxylase activity (r = 0.82).

Discussion

Efficient methods for the synthesis and analysis of diastereomers of nicotine N′-oxides and nicotine N,N'-dioxides have been developed and were used to investigate the stereoselective N-oxygenation of nicotine by human liver microsomes. In parallel studies, nicotine $\Delta^{1',5'}$-iminium ion formation (after conversion to cotinine by aldehyde oxidase) was examined to permit characterization of the monooxygenase system and the nature of the products formed. The present work shows that (*S*)-nicotine is efficiently metabolized in the presence of liver microsomes from adult humans. Like other tertiary amines, nicotine is efficiently converted to the N′-oxide and the iminium ion species. Results shown herein suggest that nicotine N′-oxide and cotinine should be major metabolites in vivo, and this has been observed (*12*). In humans, nicotine is primarily eliminated by metabolism, which is quite variable among individuals. Determination of the molecular basis for differences in the rate of nicotine metabolism may help to elucidate individual differences in detoxication, dependence liability, and smoking behavior.

The conversion of nicotine to nicotine N′-oxide was dependent on NADPH and active human liver microsomal protein and was highly sensitive to heat inactivation of the protein. Thiourea and thiobenzamide strongly inhibited nicotine N′-oxide formation. Collectively, the above findings support a role for FMO in nicotine N′-oxide formation, but they do not rule out the involvement of cytochromes P-450. Formation of nicotine N′-oxide is enhanced at pH 8.4, the pH optimum for FMO—but not cytochrome P-450—dependent reactions. As a further test of a role of FMO in nicotine N′-oxygenation, we examined the stereoselectivity of nicotine N′-oxygenation. In all cases examined, only the trans N′-oxide was observed. This result contrasts the current stereochemical view of nicotine N′-oxide formation in vivo and in vitro in animals (*30*, *44*, *45*) and man (*46*), which suggests that a considerable amount of *cis*-nicotine is formed. The basis for the stereoselectivity differences between our study and that of others is not clear. The large difference in the in vitro to in vivo ratio of human urinary metabolites arising from N′-oxygenation reported here and in the literature (*46*, *47*) was further examined by studying the stereochemistry of nicotine N′-oxide formation in humans administered (*S*)-nicotine. In all cases examined only *trans*-nicotine N′-oxide urinary metabolites were observed.[4] This result again conflicts with the reported low stereoselectivity for urinary nicotine N′-oxide observed in humans administered (*S*)-nicotine (*46*). The large difference in the results reported here and elsewhere (*46*) cannot be due only to changes in the concentration of monooxygenases in the intact liver or microsome preparations used but may possibly be due to methodology and/or autoxidation of nicotine in the analytical procedure as suggested previously (*30*). Stereoselective reduction of nicotine N′-oxide could occur in vitro and complicate the stereoselectivity assignment (*47*). If a mixture of *cis*- and *trans*-nicotine N′-oxides were formed and stereoselective reduction of *cis*-nicotine N′-oxide occurred, this would explain our result. To test this possibility, the metabolism of highly purified *cis*- and *trans*-nicotine N′-oxides and the stability of each N′-oxide diastereomer was individually examined in human liver microsomes. Using pooled human liver microsomes, no detectable conversion of either *cis*- or *trans*-nicotine N′-oxide was observed during metabolic reactions typically employed in this study. Further, highly purified *cis*- and *trans*-nicotine N′-oxide diastereomers were quantitatively extracted from microsomes or other biological

[4] S. B. Park, J. R. Cashman, P. Jacob, and N. Benowitz, unpublished results.

preparations without any detectable difference in stereochemical integrity. Because no detectable amount of nicotine N,N'-dioxide was formed during the incubations, we conclude that stereoselective oxidation, stereoselective reduction, and/or chemical isomerization of nicotine N'-oxide diastereomers does not contribute to the stereoselectivity observed for *trans*-nicotine N'-oxide formation, in vitro.

Cotinine formation was strongly dependent on NADPH and active microsomal protein. Thiourea and thiobenzamide did not inhibit cotinine formation although heat inactivation of the microsomal protein under conditions which routinely destroy 10–20% of cytochrome P-450 activity resulted in the loss of 30% of cotinine formation. Taken together, the data strongly suggest the involvement of cytochromes P-450 in the formation of nicotine $\Delta^{1',5'}$-iminium ion.

To further examine a role of FMO and cytochrome P-450 in the metabolism of nicotine, we examined selective functional markers of P-450 activity and relative FMO and P-450 levels. Nicotine N'-oxygenase activity was highly correlated with FMO form II (Table III) but not with the levels of a specific P-450 (i.e., best correlation, r = 0.27 with P-450 1A2; see ref 34). Immunoblot analyses of the same samples under similar conditions showed only a slight trace of immunoreactivity to the form I FMO from hog liver. Nicotine N'-oxide formation was not strongly correlated with gender, age, drug administration, or smoking history of the liver donor. It is likely, however, that the picture is complicated and that multiple factors contribute to nicotine N'-oxygenase activity. It is possible that both P-450 and FMO contribute to nicotine N'-oxide formation, although form II FMO, a major FMO present in adult human liver (*16*), appears to be primarily responsible for nicotine N'-oxygenation. In support of this point, no detectable amount of nicotine N'-oxide was formed from nicotine in the presence of 12 cDNA-expressed human liver P-450s (*48*). Because form I FMO possesses very low nicotine N'-oxygenation stereoselectivity (*20*) we postulate that the major monooxygenase responsible for nicotine N'-oxide formation in adult human liver is not form I FMO, but rather form II FMO.

Cotinine formation is directly correlated with 7-hydroxycoumarin formation (r = 0.80) (Table III). In addition, when the levels of six different P-450s and that of P-450 2A6 were examined (*34*), P-450 2A6 most clearly correlated with coumarin 7-hydroxylase (r = 0.91) and nicotine oxidase activities (r = 0.84). There was no clear relation among gender, age, or smoking history and cotinine formation. However, the drug administration history of the patient appears to influence 7-hydroxycoumarin and cotinine formation activity in the microsome samples examined. Thus, patients with a history of barbiturate administration had elevated coumarin 7-hydroxylase and nicotine oxidase activity. This remarkable finding is intriguing; a report describing human liver P-450 2A6 (*37*) stated that the enzyme was in primary cultures of human hepatocytes inducible by phenobarbital, and in human liver, its levels correlated strongly with coumarin 7-hydroxylase activity, all conditions observed in the nicotine oxidase activity observed in our study (Table III). Thus, cotinine formation was strongly associated with P-450 2A6, was elevated in human liver microsomes from subjects with a history of barbiturate administration, and was closely correlated with coumarin 7-hydroxylation. However, the conclusion that P-450 2A6 is largely responsible for cotinine formation does not preclude the involvement of other P-450s in this key step in human nicotine metabolism. In fact, results of another study (*48*) suggest that other human liver P-450s (i.e., 2B6, 2C9, 2E1, 2F1, and 4B1) play a prominent role in nicotine $\Delta^{1',5'}$-iminium ion formation. We cannot account for the differences in the substrate specificities of the previous study other than to point out that the human P-450 enzymes used were cDNA-expressed proteins and the present studies employed human liver microsomes.

While the human pharmacokinetics of nicotine have been extensively studied, the molecular basis for metabolism of nicotine remains unclear. Whether nicotine-derived nitrosamines are the causative agents in cancer for humans exposed to tobacco is unknown, but what is clear is that nicotine is (bio)transformed to highly reactive materials which covalently modify proteins (*5–7*) and DNA (*8–10*). Gorrod et al. (*49*) have reported a significantly elevated ratio (compared with controls) of cotinine to nicotine N'-oxide in the urine of smokers with urinary bladder cancer. In another study of cigarette smokers suffering from urinary bladder and other cancers (i.e., breast, ovary, cervix, lung, and prostate) the ratio of cotinine to nicotine N'-oxide (i.e., mean ration of 4.08) compared with controls (i.e., mean ratio of 1.7) was significantly greater (*50*). Gorrod et al. interpreted this result to suggest that decreased FMO activity thought to be primarily responsible for the conversion of nicotine to polar, readily excreted nicotine N'-oxides was associated with an increased incidence of bladder cancer (*49, 50*). It follows that, in humans, elevated cotinine levels shown to arise via aldehyde oxidase-catalyzed oxidation of P-450-formed nicotine $\Delta^{1',5'}$-iminium ions could be associated with elevated P-450 2A6 levels and bladder cancer. With the putative identification of the major human monooxygenases involved in nicotine metabolism and with the use of selective molecular probes a critical evaluation of this hypothesis can be undertaken.

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Registry No. Nicotine, 54-11-5; cotinine, 486-56-6; (*S*)-nicotine *N'*-oxide, 51095-86-4; cytochrome P-450, 9035-51-2; flavin-containing monooxygenase, 9038-14-6; (*S*)-nicotine *N,N'*-dioxide, 51744-20-8.